Serum Vaspin Concentrations Are Closely Related to Insulin Resistance, and rs77060950 at SERPINA12 Genetically Defines Distinct Group with Higher Serum Levels in Japanese Population

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Context: Vaspin is an adipokine with insulin-sensitizing effects identified from visceral adipose tissues of genetically obese rats.

Objective: We investigated genetic and nongenetic factors that define serum concentrations of vaspin.

Design, Setting and Participants: Vaspin levels were measured with RIA in Japanese subjects with normal fasting plasma glucose (NFG; n = 259) and type 2 diabetes patients (T2D; n = 275). Single nucleotide polymorphisms (SNP) at SERPINA12 (vaspin) gene locus were discovered, and five SNP were genotyped in the subjects with varied body mass index (n = 1138).

Results: The level of serum vaspin in 93% of the samples was found to vary from 0.2 to nearly 2 ng/ml in NFG subjects (n = 259) and from 0.2 to nearly 3 ng/ml in T2D patients (n = 275) (VaspinLow group), whereas a significant subpopulation (7%) in both groups displayed much higher levels of 10–40 ng/ml (VaspinHigh group). In the VaspinLow group, serum vaspin levels in T2D were significantly higher than healthy subjects (0.99 ± 0.04 vs. 0.86 ± 0.02 ng/ml; P < 0.01). Both in T2D and genotyped Japanese population, serum vaspin levels closely correlated with homeostasis model of assessment for insulin resistance rather than anthropometric parameters. By genotyping, rs77060950 tightly linked to serum vaspin levels, i.e. CC (0.6 ± 0.4 ng/ml), CA (18.4 ± 9.6 ng/ml), and AA (30.5 ± 5.1 ng/ml) (P < 2 x 10^{-16}). Putative GATA-2 and GATA-3 binding consensus site was found at rs77060950.

Conclusions: Serum vaspin levels were related to insulin resistance, and higher levels of serum vaspin in 7% of the Japanese population are closely linked to minor allele sequence (A) of rs77060950. (J Clin Endocrinol Metab 97: E1202–E1207, 2012)
obese mice ameliorated insulin resistance, indicating that vaspin is a compensatory factor in the status of obesity (1, 2). In humans, serum vaspin levels correlated with body mass index (BMI) and insulin sensitivity in adults (3–5), children (6, 7), and obese women with polycystic ovary syndrome (8–10). Furthermore, serum vaspin levels were decreased by weight reduction and lifestyle modification in obese adults (11–13) and children (6). The line of evidence supported the notion that vaspin plays an important role in the progression of obesity and insulin resistance.

Although many clinical reports investigating serum vaspin levels in the patients with obesity and metabolic syndrome are available, there are few reports investigating the genetic effects on the serum vaspin levels. Here, we measured serum vaspin levels by a wide-range RIA system, and we found that approximately 7% of the population of both normal subjects and patients with T2D demonstrated more than approximately a 10-fold higher concentration of serum vaspin levels compared with the rest of the population. We discovered that a minor allele of rs77060950 in the SERPINA12 gene was significantly associated with high serum levels of vaspin in the Japanese population and demonstrated that rs77060950 altered the transcriptional activity.

Subjects and Methods

Subjects and research design

Japanese subjects with normal fasting plasma glucose (NFG; n = 259; age, 36.6 ± 0.73 yr) and patients with T2D (n = 275; age, 61.6 ± 0.80 yr) were enrolled into this study. Healthy volunteers with NFG, who did not take any medications for diabetes, hypertension, and/or dyslipidemia, received annual medical health checkups for common disease screening at the Okayama Southern Institute of Health. NFG and T2D subjects agreed to measure serum vaspin levels. T2D subjects were treated with oral hypoglycemic agents (n = 98), insulin treatment (n = 79), or both (n = 17). All recruited T2D subjects agreed to undergo computed tomography and measure high-sensitivity C-reactive protein (hsCRP), oxidized low-density lipoprotein (LDL), leptin, adiponectin, and vaspin levels.

For single nucleotide polymorphism (SNP) genotyping of the SERPINA12 (vaspin) gene and measurement of serum vaspin levels, we further collected 1138 Japanese subjects with various BMI levels (male:female ratio, 470:668; age, 53.4 ± 12.1 yr; BMI, 27.6 kg/m²), who agreed to undergo computed tomography examinations to measure the visceral fat area (VFA) and subdermal fat area (SFA) (n = 697). We enrolled the subjects in three categories, i.e. normal weight (BMI < 25.0 kg/m²), overweight (25.0 ≤ BMI < 30.0 kg/m²), and obese (30.0 ≤ BMI kg/m²). The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the ethical committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences (Registration numbers 20, 51, and 66), Kyoto University, and also by all the appropriate institutional review boards.

Results

Serum vaspin levels well-correlated with insulin resistance

T2D subjects (n = 275; age, 61.6 ± 0.80 yr) revealed significantly higher levels of BMI, waist circumference, systolic blood pressure, plasma glucose, total and LDL-cholesterol, and lower high-density lipoprotein-cholesterol compared with NFG subjects (n = 259; age, 36.6 ± 0.73 yr) (Supplemental Table 1, published on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org). The measurement of vaspin levels in NFG subjects revealed that the majority displayed vaspin levels less than 3 ng/ml. A minor fraction (n = 18 or 6.9%) displayed much higher levels, ranging from 10 to 34 ng/ml (Fig. 1, A and B). The percentage of the population with such higher serum vaspin levels is also similar in NFG subjects, and the majority displayed vaspin levels less than 3 ng/ml; 7.2% of T2D subjects (n = 20) displayed much higher levels, ranging from 10 to 40 ng/ml (Fig. 1, C and D). By comparing the major population with vaspin levels less than 3 ng/ml (VaspinLow), vaspin levels in T2D subjects were significantly higher than NFG subjects (0.99 ± 0.04 vs. 0.86 ± 0.02 ng/ml; P < 0.01). Vaspin levels in T2D subjects were still significantly higher than NFG subjects (1.03 ± 0.06 vs. 0.83 ± 0.06 ng/ml; P = 0.038) by analysis of covariance, adjusting age as a covariant. In VaspinLow, T2D subjects, vaspin levels significantly correlated with BMI (R = 0.193; P = 0.002), SFA (R = 0.146; P = 0.016), homeostasis model of assessment for insulin resistance (HOMA-IR) (R = 0.142; P = 0.022), hsCRP (R = 0.128; P = 0.042), and leptin (R = 0.169; P = 0.019) levels, but not with VFA, oxidized LDL, and adiponectin levels. The linear regression analysis was followed by a stepwise multiple regression analysis using serum vaspin levels as the dependent variables to further analyze the significant predictors. Age, gender, BMI, SFA, VFA, Log₁₀HOMA-IR, Log₁₀hsCRP, and Log₁₀leptin levels were used as independent variables. By stepwise analysis in model 1, only Log₁₀HOMA-IR independently correlated with serum vaspin levels, and other variables demonstrating significant simple correlation with serum vaspin levels did not enter the equation at significant levels in model 2 (Supplemental Table 2).

Vaspin serum levels in VaspinHigh population are genetically defined

We next measured serum vaspin levels in samples derived from the parents of three independent VaspinHigh
subjects. All individuals from the Vaspin\textsubscript{High} group had either parent with high serum vaspin levels, suggesting an autosomal dominant or additive inheritance model (Supplemental Table 3). Genomic DNA derived from three independent Vaspin\textsubscript{High} subjects and three independent Vaspin\textsubscript{Low} subjects was used for \emph{de novo} sequencing by Illumina Genome Analyzer (Supplemental Methods). Five novel SNP unique to the Vaspin\textsubscript{high} group were identified (Fig. 1E), and we further investigated the allele frequency of these SNP and serum vaspin levels in the Japanese subjects with various BMI ($n = 1138$) (Supplemental Table 4).

Because we enrolled the normal, overweight, and obese Japanese subjects, the average BMI ($27.6 \pm 4.2$ kg/m\textsuperscript{2}) was higher, and the sample subjects may not represent the general population of Japan. This population consisted of 1028 subjects in the Vaspin\textsubscript{Low} group and 110 subjects in the Vaspin\textsubscript{High} group. In the Vaspin\textsubscript{Low} group, unlike T2D, BMI and Log\textsubscript{10}hsCRP were negatively correlated with serum vaspin levels. In contrast, Log\textsubscript{10}HOMA-IR was positively correlated with serum vaspin levels like T2D. In the Vaspin\textsubscript{High} group, BMI was the independent variable to predict the serum vaspin levels. Serum vaspin levels were higher in women than men; however, the above-mentioned correlations were similar in both men and women (Supplemental Table 5).

Among five SNP, rs77060950 most accurately predicted the serum vaspin levels, \emph{i.e.} CC ($0.6 \pm 0.4$ ng/ml), CA ($18.4 \pm 9.6$ ng/ml), and AA ($30.5 \pm 5.1$ ng/ml) (Supplemental Table 6 and Fig. 1F). The CC genotype completely matched to the Vaspin\textsubscript{Low} group (n = 1027), and CA/AA genotype corresponded to the Vaspin\textsubscript{High} group (n = 110). Thus, rs77060950 was a candidate for the functional SNP for the transcriptional regulation of vaspin gene expression, and SNP rs76624128 and rs7149693 were in the strong linkage disequilibrium with rs77060950 (Supplemental Table 6). Such strong linkage was also true in NFG and impaired fasting glucose/diabetes mellitus subgroups, defined by the fasting plasma glucose levels (Supplemental Table 7). Although serum vaspin levels were tightly associated with rs77060950, various clinical parameters obtained in the cross-sectional study were not significantly different in CC, CA, and AA genotypes of rs77060950 (Supplemental Table 8).

We found GATA transcription factor consensus binding site around rs77060950 by TFSEARCH and MOTIF search programs (Fig. 2A). We compared the luciferase activities and gel shift by using the minor (A) and major (C) alleles of rs77060950 (Supplemental Methods). Luciferase activities were significantly higher in the minor allele construct, pGL4–175A vector, compared with the major allele construct, pGL4-175C vector (Fig. 2B). Luciferase activities were higher in pGL4-295A, pGL4-

![FIG. 1. Serum vaspin levels in the subjects with NFG and the patients with T2D. A, A minor fraction (6.9%) of NFG (n = 259) displays much higher levels (circled), ranging from 10 to 34 ng/ml. B, The majority of subjects displayed vaspin levels less than 2 ng/ml. C, Similarly, 7.2% of the patients with T2D (n = 275) displayed much higher levels (circled), ranging from 10 to 40 ng/ml. D, The majority of the patients with T2D displayed vaspin levels less than 3 ng/ml. E, The localization of five SNP identified at SERPINA12 (vaspin) gene and linkage disequilibrium coefficients between five SNP in the SERPINA12 gene. F, Serum vaspin levels in the subjects with various genotypes. Among five SNP, rs77060950 completely links the serum vaspin levels, \emph{i.e.} CC ($0.6 \pm 0.4$ ng/ml), CA ($18.4 \pm 9.6$ ng/ml), and AA ($30.5 \pm 5.1$ ng/ml).]
375A, and pGL4-545A vectors compared with pGL4-295C, pGL4-375C, and pGL4-545C vectors, respectively, although they were not statistically significant. We next tested the binding of the rs77060950 minor and major allele sequences by GATA with EMSA and demonstrated DNA-protein complexes using oligo (A), minor allele sequence, and the intensity of the complexes was much weaker in oligo (C), major allele sequence (Fig. 2C). We also observed no supershifting and competition for binding by the treatment of anti-GATA-1, -GATA-2, and -GATA-3-specific antibodies in the major and minor allele sequences (Fig. 2C).

**Discussion**

A major finding of the current study is that approximately 7% of the Japanese population revealed much higher serum vaspin concentrations in both NFG and T2D subjects. Furthermore, Vaspin\textsubscript{High} subjects are genetically defined by minor allele sequence (A) of rs77060950. Another significant finding is that serum vaspin levels closely correlated with HOMA-IR rather than anthropometric parameters both in T2D subjects and the genotyped Japanese population.

Such unique logarithmic normal distribution of serum vaspin concentrations has not been reported in any other serum proteins. One exception may be the case with lipoprotein(a), which also demonstrates the logarithmic normal distribution of serum concentration. However, lipoprotein(a) is disulfide-linked to apolipoprotein B-100, and it contains two types of plasminogen-like kringle domains revealed the variation of the copy number, which resulted in the six phenotypes with varied serum concentration (14, 15). In contrast to the case with lipoprotein(a), we did not find any differences in the coding sequence of the SERPINA12 (vaspin) gene in six families of Vaspin\textsubscript{High} and Vaspin\textsubscript{Low} groups revealed by \textit{de novo} sequencing of PCR-amplified genomic DNAs (Supplemental Methods and Supplemental Table 9) using Illumina Genome Analyzer, and thus the variation of serum concentrations of vaspin could not be attributed to the difference of the protein structure and half-life of vaspin protein. Based upon these considerations, we speculated that serum vaspin levels are closely linked to transcriptional activities of the SERPINA12 (vaspin) gene, which are determined by the minor allele sequence (A) of rs77060950. Actually, minor allele sequence (A) of rs77060950 alters a GATA transcription factor binding site associated with increased luciferase activity using pGL4 construct containing 175 bp flanking

![Luciferase assay and EMSA. A, In the flanking region of rs77060950, GATA transcription factor binding site (underlined) is found, and oligos (A) and (C) were prepared (dotted underline). B, Luciferase assay using various pGL4 constructs. Higher luciferase activity was observed in pGL4–175A compared with pGL4–175C vector ($P = 0.006$). C, EMSA. The intense DNA-protein complex using oligo (A), minor allele sequence, was observed compared with oligo (C), major allele sequence (lanes 2 and 3). The DNA-protein complex was reduced by the addition of 100-fold excess of unlabeled oligo (A) (lane 7). The addition of anti-GATA-1, -GATA-2, and -GATA-3 antibodies did not demonstrate supershift (lanes 4–6).](attachment:image)
rs77060950 and enhanced the formation of DNA-protein complex by EMSA compared with minor allele sequence (C) of rs77060950. Among the GATA transcription factors, GATA-2 and GATA-3 are specifically expressed in white adipocyte precursors, and their down-regulation sets the stage for terminal differentiation. GATA-3-deficient embryonic stem cells exhibit an enhanced capacity to differentiate into adipocytes, and defective GATA-2 and GATA-3 expression is associated with obesity (16). Although TFSEARCH and MOTIF search programs predicted that GATA-2 and GATA-3 are good candidates for the regulators for transcriptional activities of the SERPINAI2 gene, the lack of supershift in EMSA by anti-GATA-2 and -GATA-3 antibodies may suggest the presence of uncharacterized transcriptional factors that bind to the genomic DNA region surrounding the rs77060950, and more intensive characterization of molecular genetics may be required.

In a previous publication, Kempf et al. (17) reported the significant association of vaspin SNP rs2236242 with T2D in the KORA F3 study, with the AA genotype bearing an increased risk [adjusted odds ratio, 2.35 (1.59, 3.46) vs. AT/TT]. However, the comparison of various clinical parameters between VaspinHigh and VaspinLow groups revealed that only insulin resistance index (microunits per milliliter) was higher in the AA genotype compared with CC and CA genotypes, although it did not reach statistically significant differences. The frequency of population with higher vaspin levels of more than 10 ng/ml seems to be different in various ethnic groups, i.e. approximately 1% in a European population in previous reports (18, 19); thus, the clinical characterization of the VaspinHigh group may be more difficult in a Caucasian population. Future cohort studies enrolling the Japanese population with higher serum vaspin levels with AA and CA genotypes of rs77060950 are required to demonstrate susceptibilities for the development of obesity, insulin resistance, diabetes, nonalcoholic fatty liver disease, and various vascular disorders.

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